University of Nevada, Reno

Dissecting a Role of Caspases in Axon Guidance: A Genetic Approach

A thesis submitted in partial fulfillment
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by

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Abstract

One of the primary mechanisms through which the developing nervous system organizes itself is known as axon guidance. In axon guidance, the axon of a growing neuron takes signaling cues from the environment to change direction on the way to its target. Guidance molecules such as netrin and slit guide growing axons towards a target. After the guidance molecules bind the cell surface receptors, a poorly understood cascade occurs which ultimately results in the cytoskeleton of the axon rearranging to allow a change in direction. This thesis proposes that the change in direction an axon undergoes after receiving guidance cues is caused by caspases (proteins known for destroying other proteins during programmed cell death.) Using genetic manipulation of fruit flies (*Drosophila Melan*ogaster), we were able to create several embryos with an increased level of caspase activity. Subsequent staining of the ventral nerve cords with the antibody BP102 revealed that simply changing the caspase activity in neurons had a profound effect on the guidance of the axons coming from those neurons. This indicates that caspases are at least partially responsible for altering the cytoskeleton of an axon and changing its direction of growth during normal neurodevelopment.

Acknowledgements

First and foremost, I want to thank Dr. Thomas Kidd. Without him, none of this would have been even remotely possible. He's been my mentor since the beginning of my research career. He took me into his lab when I knew nothing about neurobiology or lab work, and he's shown unbelievable patience with me every step of the way.

I would also like to thank all of the members of the Kidd lab for their contributions. I'd like to thank the grad students Matt Lamanuzzi, Karla Hernandez, Maryam Alvi, Logan Myers, and Adam Bousom as well as the Post Docs Elizabeth Justice, Minmin Song, And Gunnar Newquist for their mentoring and help teaching me countless aspects of research. I would also like to thank some of my fellow undergrads Tyler Detomasi, Taylor Gillis, Kirsti Walker, and Zolt Kocsis for their help around the lab with collections and crosses.

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Introduction to Axon Guidance:

This thesis looks at the effect proteins called caspases have on growing neurons in the nervous system. Before one can understand the subject of this thesis, it is important to have a basic understanding of the nervous system and the mechanisms through which it develops.

The nervous system is a network of billions of individual neurons all sending information to each other via electro-chemical signals. The individual cell unit in the nervous system is a neuron, which is composed of a round cell body, a long tube-like structure called the axon, and the synapse where the neuron connects with its target cell. Axons are unique to neurons, and are necessary for the electrochemical signaling that takes place throughout the nervous system. Proper signaling requires proper circuitry to be formed, which is dependent on axons precisely connecting to their target cell, whatever and wherever that target may be. Axon guidance is the process by which neurons accurately direct their axons to the goal. The molecules that control axon guidance are the subjects of this thesis.

Axon guidance takes place as a newly formed neuron develops. As the neuron grows it projects its axon out in a cone-shaped structure called the growth cone. As it moves along its path the growth cone follows external cues in the form of chemical signals secreted by neighboring cells. The growth cone uses these signals to determine the proper path to grow so that it may reach the end destination. The two main signal types discussed in this thesis are attractive (guiding growth towards the stimulus) and repulsive (guiding growth away from a stimulus.) As a neuron grows out its axon, the push and pull of these two guidance factors work together to guide the axon to a target. A growing axon has only these binary signals of push and pull to work with, but it manages to find its target in 3-dimensional space with remarkable precision and reliability. The neurons ability to navigate is dependent on two major steps

occurring. First a chemical signal has to be excreted from the surrounding cells in such a way that only the necessary neuron receives the signal. Then, the neuron has to integrate this information and change its subcellular machinery in such a way as to change the direction of the growth cone. This process is complex and highly reliant on the right chemicals being excreted, the machinery being rearranged properly, and several proteins mediating steps in between (proteins such as caspases.)

While the attractive and repulsive signaling occurs outside of the cell, the subcellular components inside of an axon a vital role in the axon's guidance. The growth cone is given its structure by an intracellular network known as the cytoskeleton. The cytoskeleton of the axon is composed primarily of microtubules, which are long polymers of a protein called tubulin. Microtubules are dynamic – capable of growing and shrinking in size as tubulin is either added or removed from either end of the microtubule molecule. The growth cone gets its shape from dozens of long finger-like projections called filopodia. Filopodia are composed of actin filaments, which are very similar to microtubules, only smaller. Similar to microtubules, these actin filaments can grow and/or shrink in size while they rearrange within the growth cone. This rearrangement of microtubules and filaments allows the axon to grow in length and plays a significant role in the guidance of a growth cone. (Kalil et al 2011.)

The axon changes its direction due to the rearrangement of its cellular machinery, but the axon still needs a sort of map telling it where to go. This "map" takes the form of surrounding cells excreting different guidance molecules giving directions. This process is known as chemotropism i.e. the physical movement of something that is controlled by an outside chemical stimulus. Chemotropic signals can be either attractive or repulsive. One major attractive chemotropic signal is known as netrin. Netrins were originally discovered in *C. Elegans* as part

of the gene *Unc-6*, which was heavily involved in pioneer axon growth (Hedgecock et al 1990.) The first human homolog was found a few years later (Serefini et al. 1994). Netrins were shown to cause an attractive guidance cue when bound to the cell surface receptor DCC (Harris et al. 1996; Mitchell et al. 1996). When netrins are expressed in a tissue, axons will generally grow towards that tissue, as seen in several different model organism including mice and drosophila (Barallobre et al. 2005; Andrews et al. 2008).

Slits, on the opposite hand, are class of chemotropic proteins widely known to be repulsive for axons. Robo, the primary receptor for slit, was found first in large genetic screenings of drosophila mutants (Seeger et al 1993). Without the Robo protein, axons were growing into the midline instead of their target destinations; this incorrect caused lethality in the affected embryos. Robo was known as a repulsive receptor, but the ligand that Robo bound remained a mystery. Several years later, researchers discovered the protein Slit was binding to the receptor Robo, and together they made a repulsive signal for axons. (Kidd et al. 1999). Without slit, axons were once again growing into places they should be repelled from, indicating that slit is the protein that was necessary for axon repulsion.

The current model of axon guidance suggests that the growth cone receives signals from both of these attractive and repulsive cues (netrin and slit respectively) and integrates this information into directions for movement. From the integration of these different signals, the axon "decides" where to go along its path. How the integration of external cues into axon growth directions occurs is the subject of Dr. Thomas Kidd's current research (Kidd 2009), which is the lab where I have done this thesis project. In his lab, we study what receptors guidance molecules like netrin bind, and how what that signal does, both on a cellular level and a subcellular level.

As stated before, when a molecule such as netrin binds to a receptor such as DCC in the growth cone, a signal is sent inside the cell telling it to grow its cone towards the netrin. What happens inside the cell after netrin binds is largely a mystery. We know that since the microtubules and microfilaments inside the growth cone are what provide the basic structure, these tubules and filaments need to rearrange to change the growth cones shape and ultimately the axons path. The proteins guiding this chain reaction as it occurs from cell receptor to microfilament rearrangement are some missing pieces to the complicated puzzle of axon guidance.

A large piece of this puzzle might be found in cell survival cues, also known as neurotrophic cues. During normal development the nervous system creates more neurons than it needs to function properly, and the excess neurons self-destruct through a process known as programmed cell death (PCD; Kuan et al. 2000). The most well known form of PCD is called apoptosis. Within a dying cell, apoptosis is executed and regulated by a family of proteins known as caspases. A cell that receives a signal to self-destruct activates the caspases lying within the cell. Activated caspases will then cleave many essential proteins within the cell, which ultimately leads to the cell's demise.

While caspases are essential for apoptosis, they are also involved in cell processes outside of programmed cell death. Recent studies have linked caspases to other cell functions, including axon degeneration, (Unsain et al. 2013) as well as axon guidance (Newquist et al. 2013). The Unsain research group found that when a survival cue was withdrawn from growing axons, caspases were responsible for destroying the axon. This 2013 study showed that caspases were present in the axon and functioning during neural development when neurons needed to be destroyed. The Newquist research group confirmed this involvement of caspases in dying axons,

and made an even more impressive realization: caspases were actively involved in guiding axons as they grew (Newquist et al 2013). By inhibiting caspase activity, the Newquist group changed the growth path of developing axons.

From these two papers (Newquist et al 2013; Unsain et al 2013), my thesis proposal was born. This thesis proposes that caspases are not just linked to axon growth and destruction, but are directly controlling the directional growth of axons during guidance. When a signaling molecule binds the axon, its guidance directions are carried out by activating or deactivating caspases inside of the axon. I looked to prove this by manipulating the amount of caspases in the nervous system of a fruit fly, used as a model organism. Through this work, I hope to prove this model of axon guidance set forth by Dr. Thomas Kidd (unpublished).

In this model of caspase mediated axon guidance, guidance molecules (such as netrin) are still the driving force in axon movement by binding to their specific receptors (DCC). Once a guidance molecule binds to its receptor, a protein cascade is activated. This protein cascade either activates or deactivates caspases. The activated caspases cleave the microtubules, causing a decrease in microtubules polymerization and a retraction of that growth cone portion. The deactivated caspases stop destroying microtubules, allowing them to proliferate and extend the growth cone in that direction. (See figure 1.)

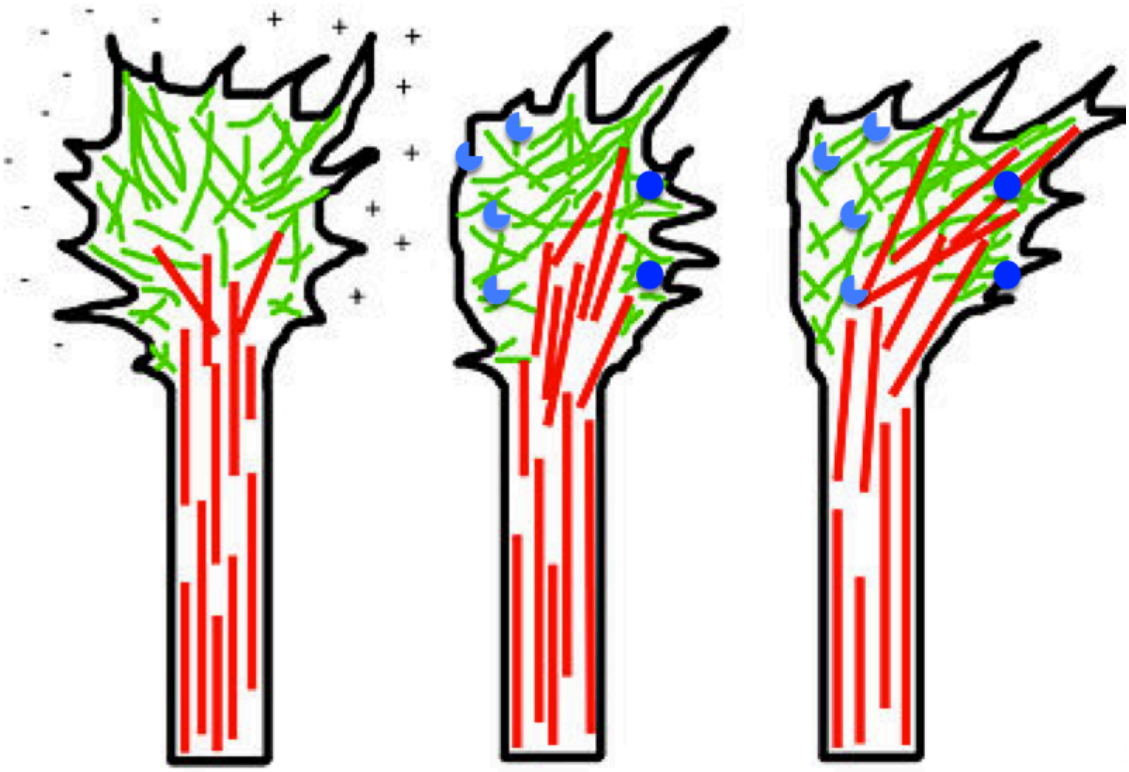


Figure 1. Model of a growing axon undergoing caspase-mediated cytoskeletal rearrangement caused by guidance molecules. The axon and its growth cone are given shape by the microfilaments (green) and microtubules (red). Attractive guidance molecules (+) are causing attraction on the right side of the cone, while repulsive molecules (-) are present on the left. Active caspases (blue circles) are present on the right side of the growth cone, causing degradation of the cytoskeletal elements and limited growth. Inactive caspases (light blue semi circles) are present on the repulsive side of the growth cone; inactive caspases do not act on the cytoskeletal elements and allow continued growth on that side. The inequality of growth causes the axon to turn right.

Axon Guidance in the Fruit Fly

To prove the hypothesis that caspase activation guides axon guidance, we first needed a model in axon guidance. Fruit flies (*Drosophila Melanogaster*) are a model organism for studying many things related to human biology, including axon guidance (Adams et al. 2000). Drosophila are eukaryotes just like humans, so they have cell types and systems that are all

similar to humans (DNA is DNA and a neuron is a neuron.) Drosophila also have many genes and proteins that are homologous to that of humans. Proteins like robo, netrin, and slit are present in both humans and flies and the proteins serve the same basic functions in both species. When dealing with systems on a basic functional level (such as axon guidance) Drosophila are more similar to us than different.

A drosophila brain obviously lacks the complexity of a human brain (both is size and function), but the Drosophila brain is still a complex organ that requires axon guidance to be formed correctly. The way this brain is formed is through axon guidance, making drosophila a great tool for studying the processes of axon guidance.

In addition to a simpler nervous system than humans, Drosophila also have more easily manipulated genetics. Drosophila have only 4 pairs of chromosomes compared to the 23 pairs a human has. Drosophila also have short generation times (7-10 days) and 1 female is capable of producing hundreds of offspring in her lifetime. These two factors allow genetic researchers like myself to rapidly turn out dozens of flies with the specific gene combination desired. Drosophila also offer unique tools for controlling genes; two such tools utilized in this thesis include balancers and the UAS Gal4 driver-expression system.

Balancers are chromosomes that inhibit gene shuffling, mainly through crossing over. The genes on these chromosomes are moved from their usual positions on the chromosomes, so any crossing over that occurs in an offspring is fatal due to gene loss and or duplication. This allows the preservation of different mutant alleles, including double and triple mutants, and the conservation of genes that are deleterious to the flies. Two commonly used balancers are Cyo and Tm6 (Casso et al 2000), which inhibit chromosomal crossing over on the second and third chromosomes respectively.

The UAS-GAL4 system allows the expression of specific genes or proteins in specific tissues of the fruit fly (Brand and Perrimon, 1993). In this system, a driver – a segment of DNA that is only expressed in a certain part of the fly is combined with a Gal4 gene. The Gal4 gene is a yeast transcript activator whose only purpose is to bind the UAS region in DNA. The UAS region of DNA is then combined with whatever gene of interest the researcher wants to have expressed. Using this UASGAL4 system, we can express proteins in specific cell types and locations such as all neurons in the central nervous system. Two such drivers that express in all neurons in the central nervous system are Scabrous (*sca*) and embryonic lethal abnormal vision (*elav*).

The arthropod version of a spinal cord is called the ventral nerve cord. Neurons begin on either side of the nerve cord. As neurons grow they extend their axons across the midline and to the other side of cord, where the axons connect with the neurons on the opposite side of the nerve cord. After crossing the midline the axons grow upwards and downwards along the longitudinals. When stained with an antibody that targets axons, (usually BP102) the ventral nerve cord displays a very identifiable ladder pattern. The rungs of the "ladder" are the commissural tracts, which are composed of the axons crossing over the midline. The sides of the "ladder" are the longitudinal tracts, which are composed of axons traveling up and down, either before or after crossing over the midline.

During the formation of these ventral nerve cords, axons are subject to both attractive and repulsive cues. The primary attractive cues are in the family called Netrins. In the fruit fly, there are two known netrin molecules – Netrin-A and Netrin-B. When netrins bind the receptor Frazzled (a fly homolog of DCC), an attractive cue is sent to the axon, causing continued growth towards the source of the netrins. Netrins also binds to the receptor Dscam and to create a similar

attractive signal. (Andrews et al., 2008) Below, figure 2 demonstrates a nerve cord and the phenotypes shown in these nerve cords when elements such as Dscam or Netrin are missing.

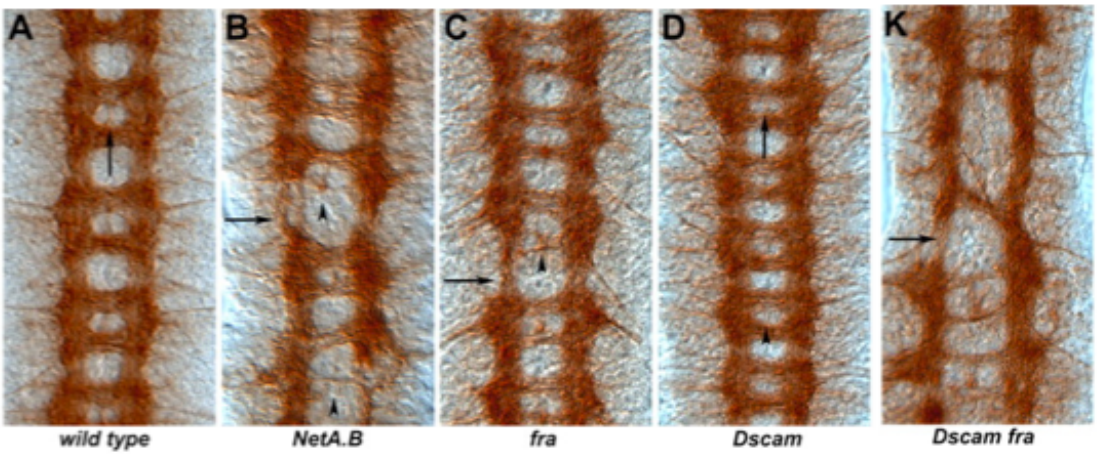


Figure 2. Images from Andrews et al. 2008 showing ventral nerve cords phenotypes of several attractive cue mutants. Wild type demonstrates the typical ladder pattern (panel A), while the mutants (panels B, C, D, and K) show aberrations in both the commissural and longitudinal tracts (indicated by arrows.) Changes in size, thickness, and position of the commissures all indicate defects in attraction across the midline.

In panel A of figure 2, a normal ventral nerve cord is demonstrated. There are two longitudinals: one on either side of the midline. The longitudinals are relatively straight, symmetrical, and thick. The nerve cord is composed of ganglia (or bundles of neurons) that form groups of four with two commissures connecting each group. These two commissures are bundled closely together, and followed by a more open area before the next pair of commissures. The arrow in panel A points to the bottom (posterior) commissure of the pair. The commissures run perpendicular to the longitudinals and parallel to each other. Any mutation in guidance will cause changes in the ventral nerve cord. If the mutation is severe enough, the changes in the nerve cord will be severe and noticeable in a BP102 dissection as shown above.

When both netrins are knocked out (figure 2 panel B) the embryos lose their primary attractive cues pulling axons across the midline. In these embryos, some of the commissures are thinner, no longer perpendicular to the longitudinals, or in several cases missing altogether. Similarly, when Fra or Dscam are missing, the phenotypes are also present due to the guidance cue having less or no receptors to bind to. The arrows in panels C and D show axon defects in *Fra* and *Dscam* mutants respectively. The most severe defects are seen in the final panel – a double mutant of both *Fra* and *Dscam*. Here the majority of the commissures are missing or thinned, and the few commissures that do exist are far from perpendicular. These flies show the worst defects in the figure. While the single mutants of a receptor *Dscam* and *fra* show slight defects, noticeable changes occur in commissural tracts when flies are missing both netrins (*netrin-AB* mutants) or both receptors (*dscam*/*fra* mutants).

Just as Netrin is the main attractant in midline crossing of the ventral nerve cord, Slit is the main repellant. Slit binds to the receptor Robo to repel axons from the midline (Kidd et al. 1999). When slit is genetically removed, the axons collapse on the midline. Similarly, when Robo is removed, axons congregate towards the midline, creating "round-a-bouts" of axons circling the midline without any repulsion to drive the axons away from the middle and towards the longitudinal tracts. All of these different phenotypes can be seen in Figure 3 below.

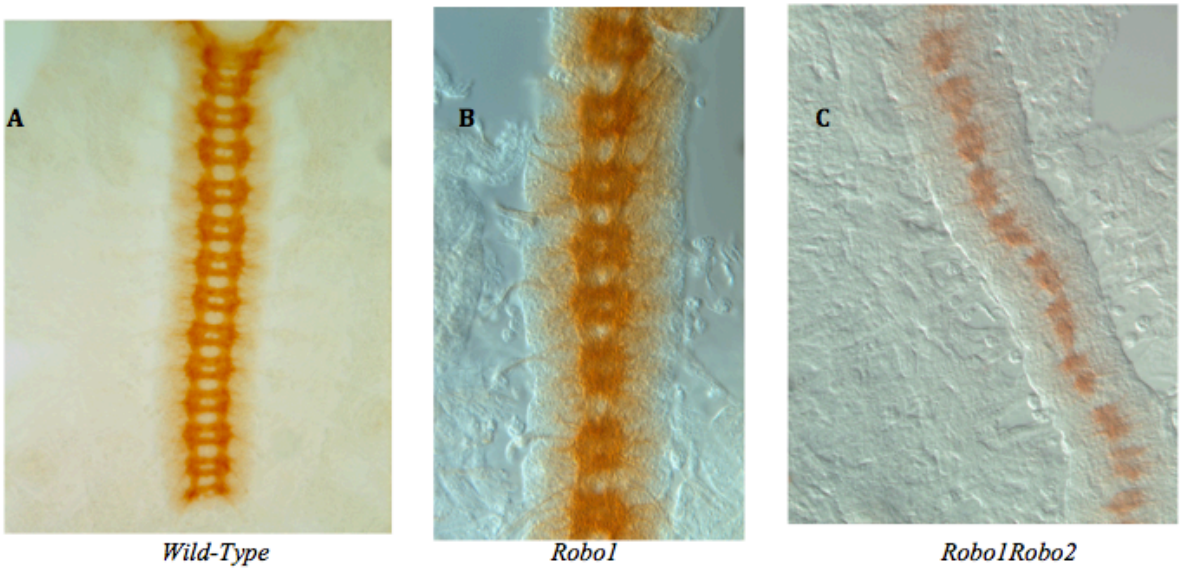


Figure 3. Images of drosophila ventral nerve cords with repellant phenotypes. (A) A typical wild-type ladder pattern. (B) A typical robo phenotype caused by a loss of *Robo1*. (C) A more severe robo phenotype caused by the absence of both *Robo1* and *Robo2*.

Figure 3 shows the effect on ventral nerve cords when the repulsive receptor robo is missing. The wild-type ventral nerve cord demonstrates axons that successfully cross the midline. The two longitudinals are a healthy distance away from the midline and the commissures show the pairing at each ganglion. Panel B, shows what happens when one of the two Robos is knocked out. Robo usually pushes axons away from the middle once they have crossed. Without Robo, the neuron bodies appear closer to the midline, and the axons are much closer together. The axons collapse around the midline, not being able to make it fully to the longitudinal on the other side of the midline. The result of this robo loss is the round circles at each ganglion. This robo pattern becomes worse when both robos are knocked out; Panel C shows a total collapse on the midline. There is no receptor for repulsion from the midline, so these axons grow to the middle and stay.

Both attraction and repulsion are vital to healthy nerve cord development. Without attraction the axons never approach the midline of the embryo. Without repulsion, the axons never leave the midline.

Caspase Activity in the Fruit fly

While attractive and repulsive molecules play a large role in guiding axons, these molecules aren't the only factors at work as the nervous system develops. A vital aspect of regulating the formation of the nervous system is cell survival factors. As the nervous system is formed, more neurons are created than are needed. The target cells that axons are growing to provide a limited amount of survival factors such as Nerve Growth Factor (Levi-Montalcini and Angeletti, 1968). Axons that don't receive this Nerve Growth Factor undergo axon degradation, where the axon is broken down in a mechanism that looks very similar to apoptosis but only affects the axon (Saxena and Caroni 2007). A recent study showed that this axon degradation was mediated by a caspase regulator XIAP (Unsain et al 2013). Furthermore, the Unsain research group showed that caspases themselves were directly involved in breaking down the axon microtubules, indicating that caspase activity can have a direct effect on axonal structure without completely killing the cell through apoptosis.

A study published last year by the Kidd research lab provided another great insight into the relationship between apoptotic signaling and axon guidance in the fly (Newquist et al. 2013). The paper showed that cell death is more prevalent in the ventral nerve cord of flies missing both Netrins A and Netrin B (known as *netAB* mutants*)*. The ventral nerve cords of these mutants showed increased nuclear fragmentation (an indicator of cell death). The n*etAB* mutants were given a viral trans gene called *p35* that blocks caspase activity. When given the *p35* gene, the

neuronal death in *netAB* mutants returned to normal levels, indicating that caspase activity was causing the cell death. Introducing p35 also rescued the guidance phenotype typically found in *netAB* mutants (Figure 4.) This indicates that caspase activity was also behind the guidance phenotype. Furthermore, when *p35* was expressed in a small sub-set of commissural axons, it was able to rescue the guidance phenotype but not the cell death. This strongly indicates that caspases could be the proteins directly changing the cell structures in the growth cone (such as microtubules) in response to guidance signals.

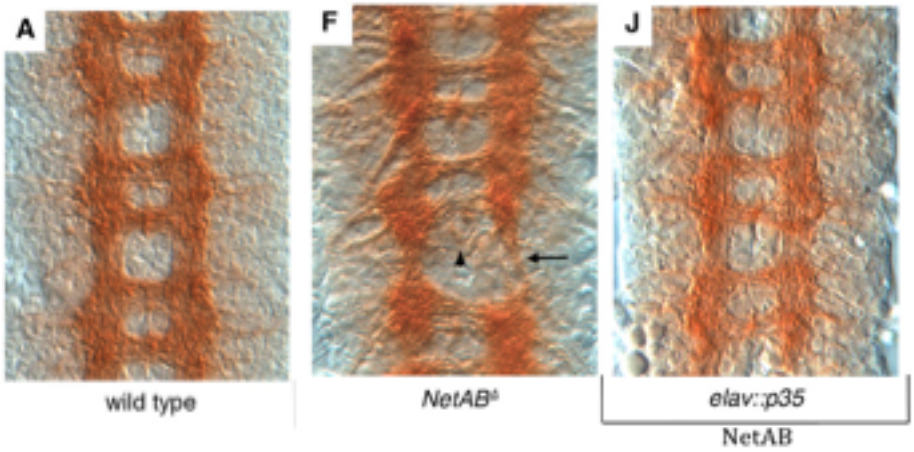


Figure 4. BP102 stains of ventral nerve cords from Newquist et al. 2013 showing caspase deactivation rescuing NetAB mutants. (A) A wild-type embryo. (F) A *Netrin AB* mutant with commissural and longitudinal defects. (J) An antiapoptotic gene *p35* driven by *elavGal4* in a *NetAB* mutant background. The *p35* gene rescues the netrin phenotype and makes the nerve cord look almost identical to the wild type. This indicates an association between caspase activity and axon guidance.

The results of Newquist et al. 2013 showed an interaction between caspases and axon guidance. To further explore this idea and provide evidence for the hypothesis in this thesis, I looked at other steps in the caspase pathway outside of the *p35* gene. Signaling for caspase activity can come from both inside the cell and outside the cell. The pathway from signaling to

caspase activation and degradation of the cell is complex and has many intermediates (see figure 5.) It would be impossible to control every aspect of this pathway, so it is important to pick out a few different proteins from different steps in the pathway and see how they have an effect on the caspase activity in the developing nervous system. From looking at the pathway, we can determine which proteins we can manipulate genetically to get an effect on the overall level of caspase activity in a cell.

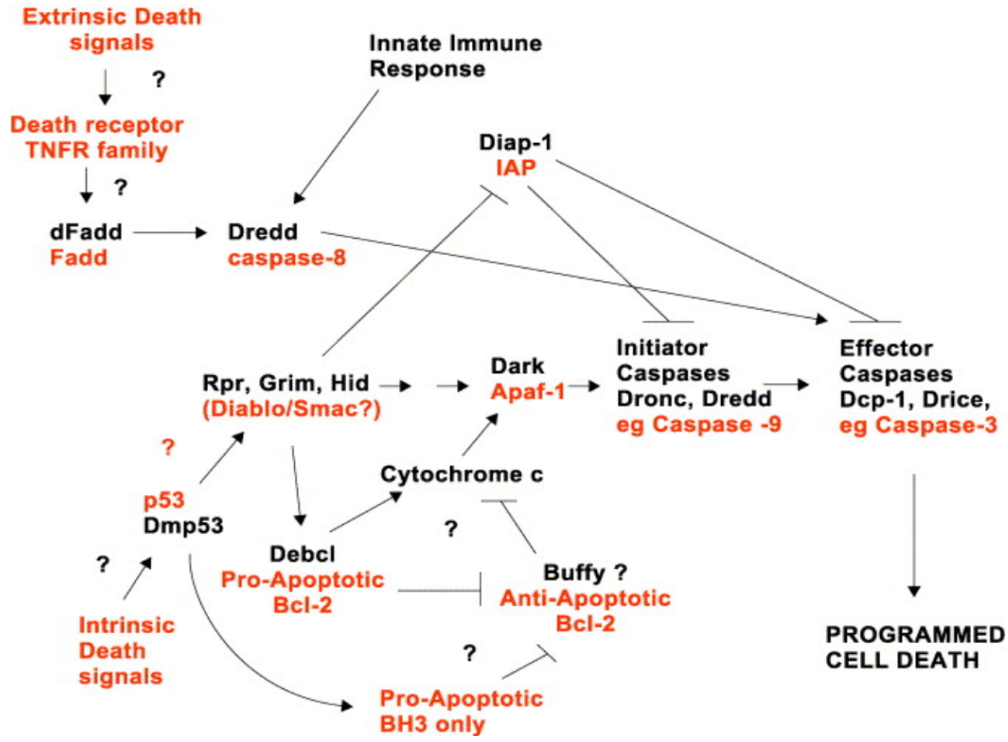


Figure 5. Caspase pathway in Drosophila taken from Richardson and Kumar 2002. Black indicates proteins found in the drosophila caspase pathway. Orange indicates human homologs of the fly proteins. Pointed arrows indicate that a protein activates the next protein in the pathway. Flat arrows indicate that the protein inhibits the next protein in the pathway.

This figure served as the main guide for this thesis. By knocking out several genes in this pathway, I sought to influence axon guidance. If axon guidance is carried out by these caspase proteins, then altering protein levels in steps of the caspase protein pathway should have noticeable effects on the development of the drosophila ventral nerve cords.

Materials and Methods:

To test caspase activity in developing neurons, I used several different Drosophila Melanogaster mutants. The Drosophila Melanogaster mutants were ordered from the Bloomington Drosophila Stock Center at Indiana University. Any fly line with a gene combination that could not be ordered from the flybase I crossed and grew myself (see the next section for more detailed information about the crosses performed.)

Once flies with correct genetic phenotypes were made, I set up a cage with different combinations of flies (called a cross.) I would take male flies of one phenotype and cross them with female flies of a different phenotype. If male flies have an example genotype of *geneA*, and female flies have an example genotype *geneB* then a cross of the two would be indicated by the following notation:

geneA (x) geneB

The offspring of this hypothetical cross would have one copy of *geneA* and one copy of *geneB* (signified with the notation *geneA*:: *geneB*.) After these flies with the correct genotypes were made and crossed, I needed to visuals their axons.

The easiest way to visualize the axons is with antibody staining. In this protocol, several hundred fly embryos from a cross are collected from an agar plate with yeast paste that the parent flies have been exposed to for 17-20 hours. The collected embryos are bleached for 5 minutes with 50% bleach in water to remove their outer most layer. After this the embryos are fixed for 15 minutes in a solution that is 1 part 3.7% formaldehyde, 1 part heptane. After the embryos have been fixed, their vitelline membrane needs to be removed. This is done by putting the embryos in a 50:50 mixture of methanol and heptane and shaking vigorously for 1 minute.

After this the embryos sink to the bottom of the methanol. These embryos are rinsed with methanol 2 more times to remove heptane, and then put into a PBS (Phosphate Buffered Saline) solution.

To prep for antibodies, the embryos are washed with PT (PBS with 0.1% Triton) 3 times for 1 minute each wash. The embryos are then blocked with 5% NGS (Normal Goat Serum) in PT for 10 minutes to prevent nonspecific binding of the primary antibody. The primary antibody is then added to the embryos and incubated overnight at 4˚ C. The main antibody we used for staining was BP102, which selectively attaches to axons in the central nervous system of embryos. The primary antibody is then washed off with PT in 3 x 1 minute washes and 3 x 10 minute washes. The embryos are then incubated for 30 minutes in a secondary antibody that binds to the primary antibody is used to amply the signal. The BP102 antibodies were created using mice, so the secondary antibody binds to mice antibodies. The secondary antibody is conjugated with HRP. HRP is an enzyme that breaks down a chemical DAB and turns it brown. The primary antibody is then washed off with PT in 3 x 1 minute washes and 3 x 10 minute washes. The embryos exposed to the secondary antibody will have HRP on the surface of their axons, so that when DAB is added (with an H202 catalyst) the axons will turn brown and become visible.

Once the nerve cords are visible after the embryos will be dissected to reveal their nerve cord, which can be photographed. Every figure in the following results sections of this thesis was obtained using this method. The embryos were stained using the methods listed above, and then I dissected the nerve cords out and photographed them using a 300x microscope.

Mutants of that are deficient in *robo* have a very specific phenotype in their ventral nerve cord caused by axons looping in circles instead of being repelled by the midline. Increasing

caspase activity should increase the repulsion if *robo* signaling is carried out by caspases. Some target lines for increasing cell death are Th^1, *UAS RPR*, and *UAS Dronc*. Th^1 is a knockout of the antiapoptotic caspase DIAP1. *UAS Dronc* is an up regulation of the initiator caspase Dronc. Rpr is a known promoter of apoptosis, which increases caspase activity. All 3 of these lines provide a different way to genetically approach increasing caspase activity at different points in the pathway. These lines will be crossed with robo mutants, and wild type flies in order to observe the effect of increased caspase activity on axon guidance. Once the crosses are made the embryos will be collected and stained as detailed above.

Genetic Crosses:

To test the involvement of caspases in axon guidance, we utilized genes from several different steps in the caspase pathway (see figure 5.) The genes we used were the genes already inserted or isolated in a fly line available at the Bloomington Drosophila Stock Center at Indiana University. The fly lines were combined in the following genetic crosses:

Cross Number 1: UAS RPR (x) ScaGal4

Reason for Cross: ScaGAL4 expresses in all neurons present in the central nervous system. The Sca gene turns on early in neuronal development and stays on throughout the life of the organism. The RPR gene a step in the caspase cascade that increases caspase activity within the cell by activating initiator caspases. Anywhere that Sca is producing Gal4, Rpr will also be made because of the UAS GAL4 system.

We predicted that increasing the RPR protein in all Neurons with the driver Sca should increase caspase signaling. The increased caspase activity would lead to a crossing phenotype

where axons are unable to cross the midline due to caspases breaking down microtubules as quickly as they are being built. The results of this cross can be seen below in Figure 6, with a wild-type embryo for comparison.

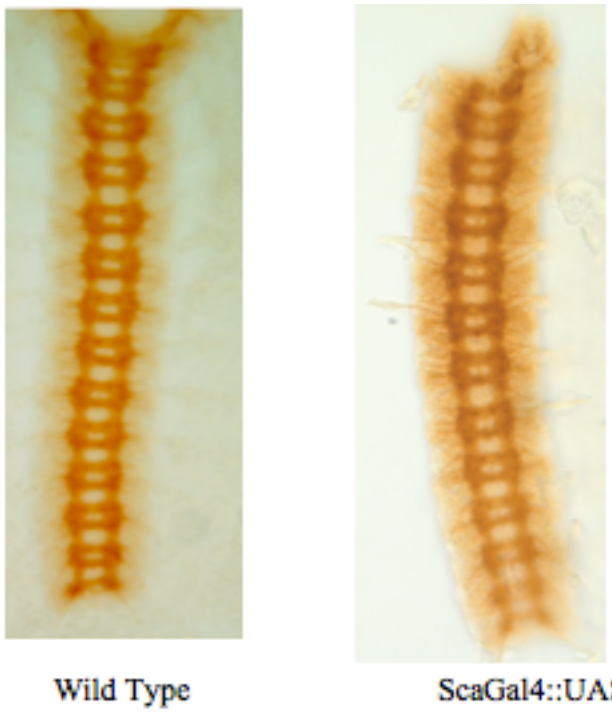
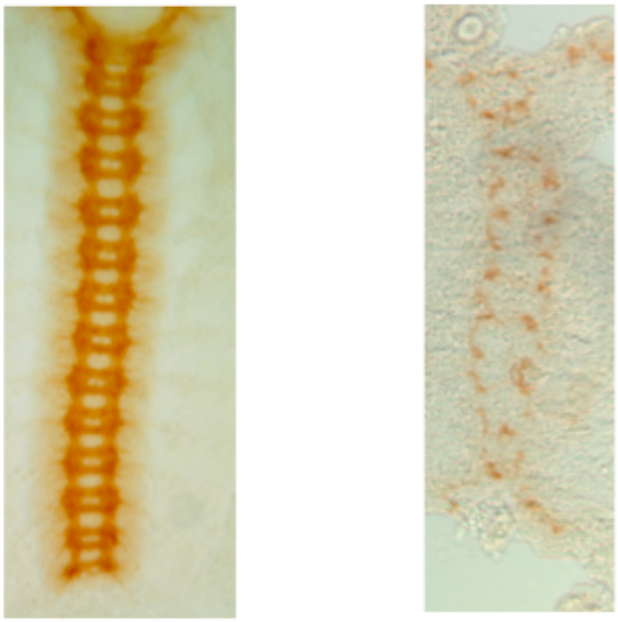


Figure 6. A ventral nerve cord staining with BP102 of a ScaGal4::UAS Rpr embryo. The first nerve cord demonstrates a normal animal with a normally developed nerve cord. The ScaGAl4::Uas Rpr fly shows no noticeable difference from the wild-type.

Results from Cross 1: Although it was expected that ScaGal4::UAS Rpr would cause a noticeable phenotype, the results show otherwise. There is no noticeable difference between the two. This indicates that either the experiment didn't work properly, or our hypothesis was incorrect. To further test our hypothesis, we used a different driver (cross 2).

Cross Number 2: UAS RPR (x) ElavGal4

Reason for Cross: The first cross displayed no noticeable results. To try and get a noticeable result, I set up a nearly identical cross using a different driver. The ElavGa4 driver expresses strongly in all neurons. However, ElavGal4 activates later in the development of the embryo than ScaGal4, and it does not activate quite as many neurons as ScaGal4. The UAS Rpr gene means any cell activated by ElavGAl4 will express Rpr and therefore should have increased caspase activity. The results of this cross can be seen below in Figure 7, with a wild-type embryo for comparison.



Wild Type ElavGal4::UAS Rpr

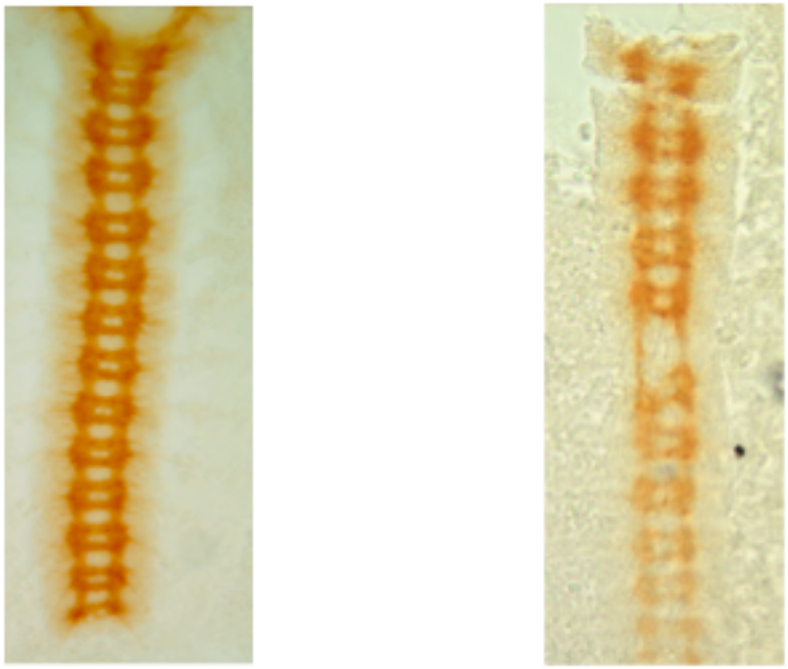
Figure 7. A ventral nerve cord staining with BP102 of an ElavGal4::UAS Rpr embryo. The first nerve cord demonstrates a normal animal with a normally developed nerve cord. The ScaGAl4::Uas Rpr fly shows a drastic difference from the wild type. The typical latter pattern is completely destroyed. Axons are missing from both the longitudinals and commissures, indicating that guidance is massively hindered by caspase activity.

Results from Cross 2: In cross 1 the ScaGal4::UAS Rpr flies showed no noticeable phenotype. In this cross (ElavGal4::Uas Rpr) the phenotype is the exact opposite. The phenotype in these flies

is drastic and unmistakable. Every single commissure in the ventral nerve cord is either missing or thinned. Every longitudinal is also missing or thinned. The phenotype in these flies is so severe it is hard to gain any more knowledge about the axon crossing, other than the fact that increasing Rpr in these animals with the Elav driver completely hinders axon guidance. To find a slightly less severe phenotype, a different caspase activator was used in cross number 3.

Cross Number 3: UAS Dronc (x) ElavGal4

Reason for Cross: Rpr caused an increase in caspase activity, but it caused to great of an increase when paired with Elav. To try and turn down the volume on this caspase activity in the nervous system, a different gene in the caspase pathway (figure 5) was used. The gene we decided on was Dronc. Dronc is a pro apoptotic caspase pathway that is downstream of Rpr. The Dronc gene was paired with the same ElavGal4 from cross 2. The cross of these Elav and Dronc genes was expected to cause increased caspase activity in all neurons expressing Elav. The results can be seen below in figure 8.



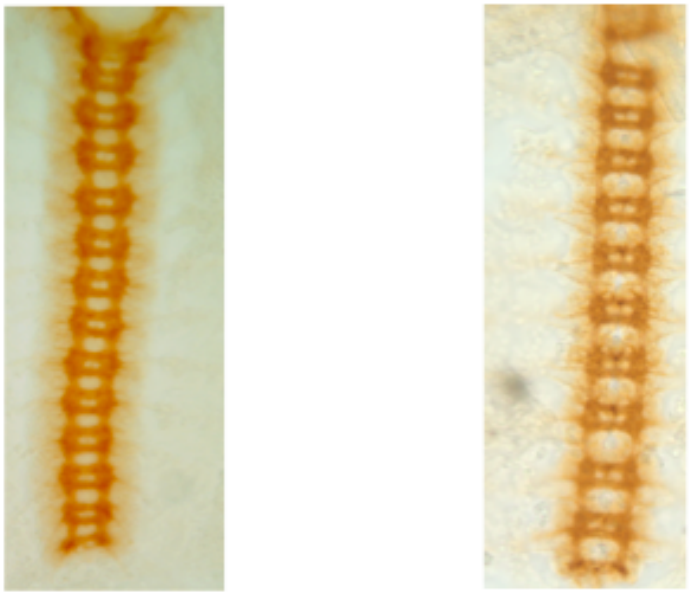
Wild Type ElavGal4::UAS Dronc

Figure 8. A ventral nerve cord staining with BP102 of an ElavGal4::UAS Dronc embryo. The first nerve cord demonstrates a normal animal with a normally developed nerve cord. The ScaGAl4::Uas Dronc fly differs from the wild type. The typical latter pattern is mostly present, but in the middle of the cord there is a noticeable gap. Part of the commissural axon crossing from the right to the left is missing, which is what causes this gap.

Results from Cross 3: In this cross (ElavGal4::Uas Dronc) a clear phenotype can be observed. The commissure is missing in the middle, which causes the huge gap in the ladder pattern. This missing commissure indicates that the commissural axon isn't crossing the midline.

Cross Number 4: UAS Dronc (x) ScaGal4

Reason for Cross: Elav Gal4 caused a phenotype with both UAS Rpr and UAS Dronc. Sca Gal4 didn't cause a phenotype with UAS Rpr. For the sake of making a control, UAS Dronc was crossed with Sca Gal4 to see if a phenotype could be observed or not. The results can be seen below in figure 9.



Wild Type ScaGal4::UAS Dronc

Figure 9. A ventral nerve cord staining with BP102 of a ScaGal4::UAS Dronc embryo. The first nerve cord demonstrates a normal animal with a normally developed nerve cord. The ScaGAl4::Uas Dronc fly is not noticeably different from the wild-type fly.

Results from Cross 4: Although it was expected that ScaGal4::UAS Dronc would cause a noticeable phenotype, there is no noticeable difference between the mutant and a wild-type fly.

Cross Number 5: Diap1 knockout Tau/Cyowg ; Th4/Tm6Hz (x) Tau/Cyowg ; Th4/Tm6Hz

Reason for Cross: Th4 is another name for DIAP1, which is an antiapoptotic protein that inhibits caspase activity. A knockout in this gene should cause an increase in caspase activity throughout the embryo, including the nervous system. Figure 6 shows several dissections of embryos with this increased caspase activity.

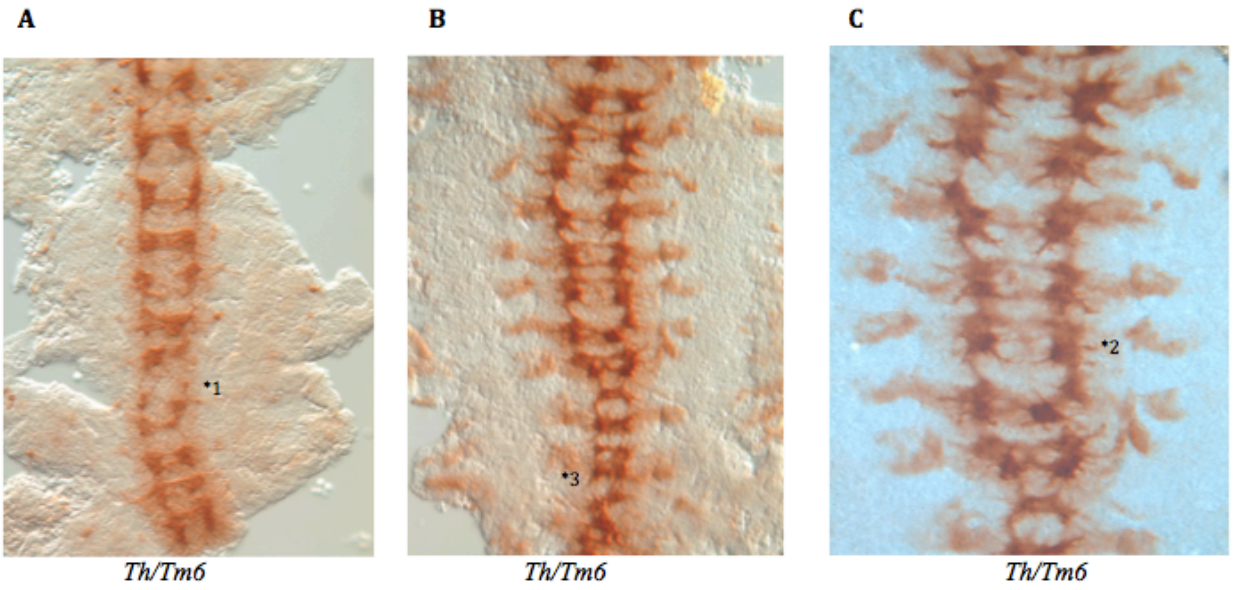


Figure 10. A ventral nerve cord staining with BP102 of a Diap1 knockout. Embryonic ventral nerve cords stained with BP102**.** Panel A shows a Diap1 knockout mutant over the balancer TM6. Panel B shows another Diap1 knockout mutant over the balancer TM6. Panel C shows a higher magnification of embryo B.

Results from Cross 5: From looking at these photographs, it is clear that both embryos demonstrate an extreme phenotype. Almost every commissure is missing or thinned (see the *1 on the figure). Under higher magnification, it's clear that the neurons are growing axons, but the axons aren't traveling to their destination. The axons stall out in the midline (*2). The neurons themselves also show collapsing onto the midline (*3).

Cross Number 6: robo robo2/Cyo ; UAS Rpr (x) Robo robo2/cyo ; Fitz Gal4

Reason for Cross: The double Robo mutant shows an extreme phenotype with the axons all congregating in the midline and not leaving, similar to a slit mutant. We hypothesize this is due to a decrease in caspase signaling. Ftz Gal4 and UAS Rpr should increase caspase signaling in a small subset of these neurons, and hopefully this will rescue the phenotype. It might rescue the phenotype completely, partially (making it look more like a single Robo mutant perhaps, or something in between), or not at all. Even if it did not rescue, that could have been an indication that the caspase activity was not strong enough to rescue the lack of signaling found in robo1robo2 mutants. It is was a possibility that the caspase signaling will override the robo deficiency completely, and then the phenotype would look similar to the results from cross 2.

Robo mutants are known for the axonal and neuronal collapse on the midline caused by a lack of repulsion. We hypothesized that this midline collapse was caused by a lack of caspase activity driving the axons away from the midline. In the cross, we took mutants missing both Robos (*Robo1Robo2)* and expressed caspase activator Rpr with the neuronal driver Ftz. The results can be seen in Figure 11.

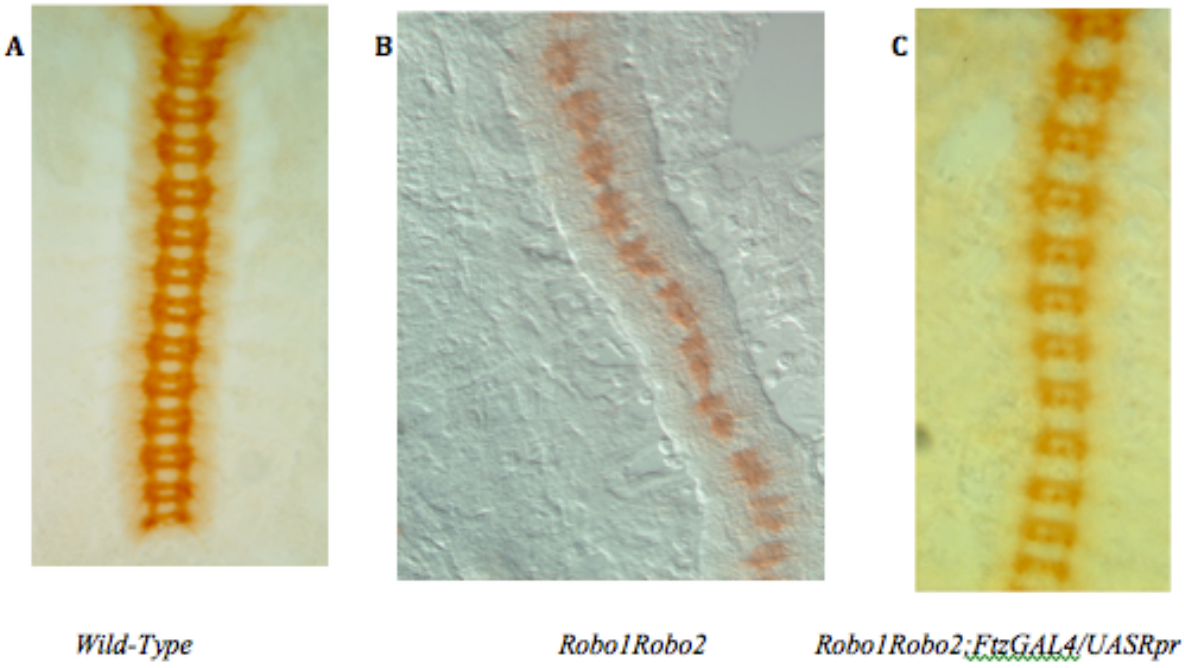


Figure 11. An increase of caspase activity in robo mutants causes a rescue of the robo phenotype. Embryonic ventral nerve cords stained with BP102**.** (A) A wild-type embryo showing a normal (B) An embryo missing both robo1 and robo2. These embryos are characterized by total collapse of axons and neurons upon the midline (C) A robo1robo2 mutant with UAS Rpr being driven by FtzGal4. The embryo demonstrates a complete rescue as it resembles the wild-type embryo.

Results from Cross 6: A typical Robo1 Robo 2 mutant has an extreme Robo phenotype caused by axons collapsing on the midline as seen in panel B. The FtzGAl4 cross with Rpr caused an interesting result. The ventral nerve cord in these flies shows no difference from that of a wild-type, suggesting that this cross completely rescued the robo1robo2 phenotype.

Discussion

The crosses performed in this experiment all support the hypothesis that caspase activity modulates axon guidance. As previously demonstrated in Newquist et al. 2013, blocking caspase

activity was able to rescue Netrin axon guidance phenotypes. In the Newquist paper, the researchers believed *p35* rescued the netrin phenotype by inhibiting caspase activity. This implies that an increase in caspase activity was the cause of the original crossing phenotype. The results from our experiments support this observation. However, instead of blocking caspase activity with *p35*, our experiments looked to increase the caspase activity.

When caspase activity was increased pan-neuronally using Elav and either Rpr or Dronc, the phenotypes resembled that of a *Netrin AB* or *Dscam/Fra* mutant. These results compliment those of the Newquist et al research group. In their paper, they showed that blocking caspases fixed the crossing phenotype in *NetrinAB* mutants. In our experiment we created a phenotype similar to a *NetAB* phenotype by increasing caspase activity in an otherwise normal fly. If the attractive Net AB signal typically inhibits caspase activity in the growth cone, then flies lacking this attractive signaling should also lack caspase inhibition. By increasing caspase activity in neurons with Rpr, we are simulating a lack of attractive signaling causing inhibition, so the phenotype resembles that of an attractive mutant. The axons will behave as if they are *netrin* or *dscam*/*fra* mutants because the regular attractive signaling trying to inhibit caspases is being ignored. The axons fail to cross the midline even though Netrin and Dscam/fra are still being expressed at normal levels.

During normal neuron development, a basal level of caspase activity is present (Unsain et al., 2013.) The Rpr and Dronc experiments showed that increasing caspase activity in the neuron inhibits crossing in the midline. To further prove this, we looked to increase caspase activity through yet another step in the pathway. We used the Diap1 knockout to increase caspase activity in the entire animal. This increased caspase activity no doubt has effects on every system, but we were looking only at the nervous system. When caspase activity was increased

throughout the entire animal (Diap1 knockout), the ventral nerve cord phenotype still resembled that of an attractive mutant. This is further evidence to support the idea that caspase activity regulations axon growth.

More impressively, it is clear that the axons of the neurons are growing and not just dying. When increasing caspase activity in every cell, a legitimate concern is that the cells could just go into full-blown apoptosis caused by the elevated caspase levels. However, as seen in figure 10, each neuronal ganglion appears to be present and the ganglions all have several hair-like projections coming from them. These projections are the axons, and we can see they are trying to grow, but appear to be stalling. In a wild-type fly at the same stage, these axons have already made their journey across the midline. In these flies, the axons haven't died, but they haven't moved much either. This is evidence that the increased caspase activity is causing the axons to break down as fast as they are being made, effectively causing a treadmill effect with no real growth. When paired with the Rpr and Dronc over-expressions, the Diap1 knockout suggests that increasing caspase activity negatively affects midline crossing.

Increased caspase activity mimics repulsion from the midline even in the presence of attractive cues. Therefore, if the same caspase activators are placed in a mutant with defective repulsive cues, the caspase activity should rescue the lack of repulsion. *Robo* mutants lack repulsion in the midline because slit cannot signal the axons to grow away from the midline through the activation of localized caspases. This *robo* phenotype of axons circling the midline was completely rescued when increased caspase activity was artificially restored in figure 11. The neurons are completely missing the two major repulsive receptors, but are still managing to be repelled from the midline. This demonstrates that caspase signaling is directly causing changes in axon repulsion; repulsion can be caused with just caspase activity alone.

The results discussed above all support the hypothesis that caspase activity is playing a significant role axon guidance. However, one part of the experiment did not produce the expected results. The Sca driven expression of Rpr and Dronc in the neurons did not produce a phenotype. Instead these embryos appeared to be wild type. The Sca driver should have expressed Rpr and Dronc in a very similar manner to the elav driver. This discrepancy has no explanation at the time, but a few different scenarios could explain these peculiar results. It is possible that the stock of ScaGal4 was not working effectively. This explanation is the easiest to test by repeating the cross with a different ScaGal4 line. It is known that ScaGal4 expression takes place at a different stage in development. It is therefore possible that the small difference in the time and place of expression caused by ScaGal4 is significant enough to change the phenotype of the animal. If caspases were only expressed after the axon has crossed the midline, then a crossing phenotype would not be observed. It is also possible that the ElavGal4 line is interacting in an unpredictable way with the embryo, causing a false phenotype. This however is the least likely, since the group in Newquist et al. used the ElavGal4 line to drive their expression. These possibilities need to be further explored with more experiments. A third driver that expresses in a smaller set of neurons could be used, possibly FtzGal4. Driving expression with a third driver would help determine which of the other two drivers is defective. Given the results from the Diap1 knockout and Rpr expression in RoboRobo2, we still feel confident in our hypothesis that caspase activation is what is causing the observed phenotypes.

When combined with the results from Newquist et al. 2013, the results of this thesis make a strong argument that attractive and repulsive cues cause a change in caspase activity, which in turn directly controls axon guidance. The Newquist paper demonstrates that blocking caspase activity counteracts the lack of attractive cues. This thesis shows that increasing caspase activity

mimics a loss of attractive cues. All of our experiments all suggest that increasing caspase activity within a cell will cause repulsion, and decreasing caspase activity will cause attraction.

Further experiments should be done to prove that our experiments are using localized subcellular caspase activity to control axon guidance. While looking at the nerve cords of embryos can give extremely valuable information, it has its limits.

The results of this thesis could have great significance in the field of neuroscience. Axon guidance is far more complex than the scope of this thesis, so any contribution to pathways and interactions in axon guidance can help neuroscientists fully understand how something as complex as the human brain can be formed. The involvement of caspases in shaping microtubules in response to guidance molecules is just a small piece of the massive puzzle that is neurodevelopment.

On a more grounded level, axon guidance has long been a subject of interest when dealing with spinal cord injuries. When a person breaks their spinal cord, that person is typically left with total paralysis below the point of injury. The injured person loses all feeling and motor control because the nerves in the lower body are unable to connect to the brain due to the break in the spinal cord. If the nerves in the spinal cord could be induced to grow again, a spinal cord injury could be corrected with new axons growing and forming connections where the old axons used to be. Factors like Netrin could be injected into the site to stimulate nerve growth. Now that we know that netrins inhibit caspases, it is possible that inserting netrin into the site might also decrease the caspase activity in the area. Decreasing caspase activity at the site could prove useful immediately after an injury when axons are degrading. It might also help new axons grow. Using guidance molecules to fix spinal cord injures might seem like a lofty and far-fetched goal,

but the research done in this thesis could lay the basic ground-work for researchers of the future. With our knowledge of caspase modulation of axon guidance, these future researchers could uncover how the remarkably complex human brain is formed from nothing but a network of individual neurons connecting to other neurons.

References:

Adams, M.D. et al. (2000) The genome sequence of Drosophila Melanogaster. *Science* 287: 2185-95.

Andrews, G.L., Tanglao, S., Farmer, W.T., Morin, S., Brotman, S., Berberoglu, M. A., Price, H., Fernandez, G.C., Mastick, G.S., Charron, F., And Kidd, T. (2008) Dscam guides embryonic axons by Netrin-dependent and -independent functions. *Development* 135: 3839–3848.

Barallobre, M.J., Pascualc, M., Del Rio, J.A., and Soriano, E. (2005) The Netrin family of guidance factors: emphasis on Netrin-1 signalling. *Brain Research Reviews* 49: 22–47

Casso, D., Ramírez-Weber, F., and Kornberg, T.B. (March 2000). GFP-tagged balancer chromosomes for Drosophila melanogaster. *Mechanisms of Development* 91 (1-2): 451–454.

Harris, R., Sabatelli, L.M., and Seeger, M.A. (1996) Guidance cues at the Drosophila CNS midline: Identification and characterization of two Drosophila Netrin/UNC-6 homologs. *Neuron* 17: 217–218.

Hedgecock, E., Culotti, J., and Hall, D. (1990) The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in C. elegans. *Neuron* 4 (1) 61-85.

Kalil K., Li, L., and Hutchins, B.I. (2011) Signaling mechanisms in cortical axon growth, guidance, and branching. *Frontiers in Neuroanatomy* 5: 62.

Kidd, T. Model of axon guidance under caspase control. Unpublished Manuscript. University of Nevada, Reno.

Kidd, T. (2009). Neuroscience: crossing the line. *Science* 324:893-894.

Kidd, T., Brose, K., Mitchell, K. J., Fetter, R. D., Tessier-Lavigne, M., Goodman, C. S. and Tear, G. (1999) Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. *Cell* 92: 205-215.

Kuan, C.Y., Roth, K.A., Flavell, R.A., and Rakic, P. (2000) Mechanisms of programmed cell death in the developing brain. *Trends in Neuroscience* 7: 291-297.

Levi-Montalcini, R., and Angeletti, R.U. (1968) Nerve growth factor. *Physiological Reviews* 48: 534-569.

Mitchell, K.J., Doyle, J.L., Serafni, T., Kennedy, T.E., Tessier-Lavigne, M., Goodman, C.S., and Dickinson, B.J. (1996) Genetic analysis of Netrin genes in Drosophila: netrins guide CNS commissural axons and peripheral motor axons. *Neuron* 17: 203 – 215.

Newquist G, et al. (2013) Blocking apoptotic signaling rescues axon guidance in Netrin mutants. *Cell Rep*orts 3: 595-606.

Nicolas Unsain, N., Higgins, J., Parker, K. N., Johnstone, A. D., Barker, P. A. (2013) XIAP Regulates Caspase Activity in Degenerating Axons. *Cell Reports* 4(4): 751-763.

Richardson, H., Kumar, S. (2002). Death to flies: Drosophila as a model system to study programmed cell death. *Journal of Immunolical Methods* 265(2): 21-38.

Saxena, S., Caroni P. (2007) Mechanisms of axon degeneration: from development to disease. *Progress in Neurobiology* 3: 174-91.

Serafini, T., Kennedy, T. E., Galko, M. J., Mirzayan, C., Jessell, T. M. and Tessier-Lavigne, M. (1994) The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. *Cell* 78: 409-424.

Seeger, M., Tear, G., Ferres, M.D., Goodman, C.S. (1993) Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline. *Neuron* 10(3): 409-426.

Tessier-Lavigne, M., and Goodman, C. S. (1996). The Molecular Biology of Axon Guidance. *Science* 274: 1123-133.

Unsain, N., J.M. Higgins, K.N. Parker, A.D. Johnstone, and P.A. Barker. (2013) XIAP regulates caspase activity in degenerating axons. *Cell Reports* 4: 751–763.